Effect of nitric-oxide-generating system on microcirculatory blood flow in skin of patients with severe Raynaud's syndrome: a randomised trial

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Summary

Background Patients with Raynaud's syndrome have abnormal digital vasoconstriction, which may be secondary to impaired synthesis of, or impaired sensitivity to, nitric oxide. We studied the effect on microcirculation of a nitric-oxide-generating system applied topically to the finger and forearm of healthy volunteers and patients with primary Raynaud's syndrome.

Methods We did a single-blind, randomised, placebo-controlled, cross-over study of the microcirculatory response to topical application of a nitric-oxide-generating gel in 20 patients with severe Raynaud's syndrome, and ten healthy volunteers. We prepared the nitric-oxide-generating system by mixing a solution of KY jelly and sodium nitrite (5% weight/volume), with a solution of KY jelly and ascorbic acid (5% weight/volume). About 0·5 mL of each solution was separately applied to the skin of the forearm (3 cm²), and then mixed with a sterile cotton bud. A similar procedure was done simultaneously on the other arm with KY jelly only (placebo). The procedure was then repeated on the finger pulps. Changes in skin microcirculatory volume and flux were measured bilaterally by infrared photoplethysmography and laser doppler fluxmetry, respectively.

Findings In the forearm, blood flow increased significantly after application of the active gel both in patients with Raynaud's syndrome (microcirculatory volume from mean area under the curve 98 [SE 14] to 1024 [130]; microcirculatory flux from 5060 [462] to 74 800 [3940]) and in healthy controls (volume from 85 [19] to 1020 [60]; flux from 4420 [435] to 84 500 [7000]). In the fingers, although baseline blood flow was lower in patients than in controls, both groups showed increases with application of active gel (volume from 1100 [194] to 3280 [672] and 2380 [441] to 6160 [1160], respectively; flux from 33 400 [4200] to 108 000 [13 600] and 52 000 [8950] to 185 000 [19 500]). Increases in blood flow with placebo gel were not significant. No adverse effects were reported.

Interpretation In primary Raynaud's syndrome, topical application of a nitric-oxide-generating system can stimulate an increase in both microcirculatory volume and flux.

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See Commentary page ???

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Introduction

In Raynaud's syndrome, circulatory changes occur in the fingers in response to cold or stress. Affected digits become white (digital vasospasm), followed by blue (capillary stagnation), then red (reactive hyperaemia). This syndrome can be primary or, less commonly, secondary in association with connective-tissue diseases such as scleroderma and systemic lupus erythematosus. Some cases are familial.[1] Between 3% and 20% of adults are affected,[2] more women than men (ten/one ratio). Various interventions and treatments, including cessation of smoking, keeping warm, and administration of slow-acting calcium-channel-blocking agents and derivatives of nicotinic acid, are used but some have intrusive side-effects, and the effectiveness varies.[3]

The cause of primary and secondary Raynaud's syndrome is still poorly understood. However, evidence has emerged to support the "local fault" theory of Lewis[4] since abnormalities in adrenergic control of vascular tone have been found.[5] Other mechanisms have been proposed, including abnormalities in vascular endothelium and platelet aggregation.[6] A defect in synthesis of nitric oxide has also been described.[7]

Endothelial dysfunction is recognised as playing an important part in the pathogenesis of peripheral vascular diseases, including Raynaud's syndrome.[8] Several pathogenetic mechanisms for endothelial dysfunction have been proposed, such as abnormalities in signal transduction, and decreased synthesis and accelerated inactivation of endothelium-derived relaxing factor (nitric oxide).[9] Nitric oxide is a potent vasodilator,[10] that is synthesised and released by vascular endothelial cells, and has an important role in the regulation of vascular local resistance and blood flow. Organic nitrate cream that generates nitric oxide is available for use in angina (Percutol, Dominion, Surrey, UK), but the cutaneous vasodilatory effect is transient, and attempts to use it for the treatment of Raynaud's syndrome have been limited by systemic side-effects including headache.

Nitric oxide can be generated by mixing sodium nitrite with ascorbic acid (vitamin C).[11] This mixture generates nitric oxide and dehydrosodium ascorbate. Preliminary studies have shown that such a topical preparation is well tolerated, and may produce a 490% increase in skin blood flow in the forearm with no increase in skin temperature.[12] We have shown that nitric oxide is continually released from the surface of the skin by a mechanism that appears to be independent of nitric oxide synthase.[13] Nitrate excreted in sweat is reduced to nitrite by an unknown mechanism, which may involve nitrate reductase expressed by skin commensal bacteria. Alternatively, mammalian nitrate reductase may be present in the skin.[14] Nitrite is rapidly reduced to nitric oxide on the skin surface, perhaps because the sweat is acidic and because reducing substances such as ascorbic acid are present. Although the amount of nitric oxide generated by this physiological mechanism is not sufficient to affect skin blood flow, very

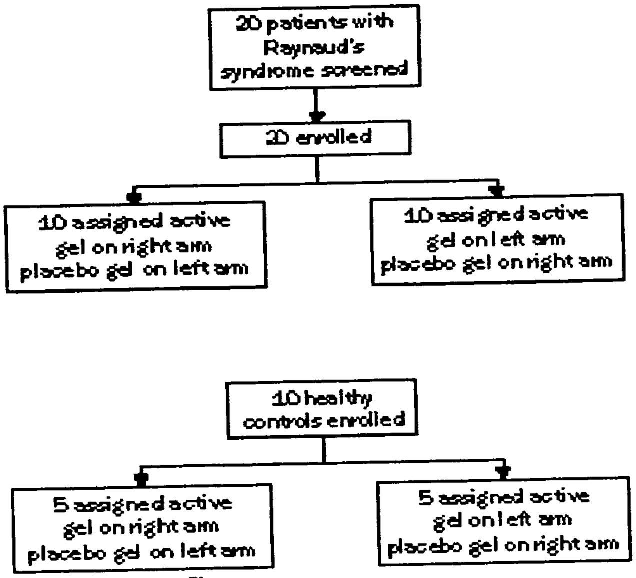

Figure 1: **Trial profile**

large amounts of nitric oxide can be generated by the topical application of nitrite and ascorbic acid.

The main aim of this study was to use non-invasive vascular measurement techniques to assess the effects on the skin microcirculation of a topically applied nitric-oxide-generating system in patients with severe Raynaud's syndrome and in healthy individuals. The techniques of non-invasive measurement have been previously used to study skin microcirculation in other disorders.[15]

Patients and methods

Patients

We did two studies: a preliminary dose-ranging study in ten healthy volunteers, and a single-blind, randomised, placebo-controlled, cross-over study in 20 normotensive and otherwise healthy patients with severe primary vasospasm (Raynaud's syndrome) with no organic vessel-wall changes. Severe Raynaud's syndrome was defined by the criteria of Allen and Brown,[16] with five or more attacks during 1 day or ten attacks per week during the winter, each lasting for more than 30 min. The severity of the vasospasticity was confirmed objectively by microcircular measurement after a cold pressor test (immersion of the hand for 1 min in a water-bath maintained at 20°C).[17] Patients with connective-tissue disease were excluded before entry into the study by nail-fold capillaroscopy of all digits and an autoantibody screen. Each patient had a normal and sustained vasodilatory response to a warm stress test (immersion of the hand for 5 min in a water-bath maintained at 40°C), measured by mean hand temperature and microcirculatory blood flow.[18] Four of the patients smoked, but they abstained for 24 h before participation in the study. Additionally, ten healthy, non-smoking normotensive, normocholesterolaemic, normoglycaemic controls with no evidence or history of autoimmune or vascular disease were recruited for comparison.

Concomitant disease, current drug therapy, and history of tobacco consumption were recorded. Body-mass index was calculated. Exclusion criteria were any significant concomitant disease; pregnancy or breastfeeding; history of drug allergy; eczema, psoriasis, or cracks or ulceration of the fingers; treatment with drugs with known or potential activity on the cardiovascular system or on blood rheology (eg, angiotensin-converting-enzyme inhibitors, calcium-channel blockers, aspirin, or any other non-steroidal anti-inflammatory drugs); blood pressure more than 160 mm Hg systolic or more than 100 mm Hg diastolic; cardiac arrhythmias other than supraventricular extrasystoles; and failure to show the normal and sustained vasodilatory response to a warm stress test of the hands. Abnormal responses to warming are a reflection of structural change and of autonomic and endothelial dysfunction.[18]

	Patients with severe Raynaud's syndrome	Controls
Sex	17/3	7/3
Mean (SE; range) age (years)	39·1 (2·5; 17–61)*	29·5 (1·8; 24–38)
Mean (SE; range) body-mass index	23·7 (0·8; 18·5–35·1	24·3 (1·4; 18·9–32·3)

*p<0·005 versus healthy controls.

Table 1: **Baseline characteristics**

Patients gave informed written consent, and the study was approved by the East London and City Health Authority Ethics Committee.

Methods

All examinations were done in a quiet, draught-free laboratory, controlled for temperature and humidity (24°C±1°C; relative humidity 30–40%), in the morning at about the same time of day for each patient during the winter of 1996–97. All patients had had a light breakfast avoiding fatty foods and caffeine, and had abstained from vigorous exercise since the previous evening.

Microvascular assessment of the peripheral blood flow in the arm was by use of standard techniques, which have been extensively described.[15,17–21] All investigations were non-invasive and painless, and were done bilaterally with the patients sitting with their forearms supine and supported on a frame at an angle of 30° to the horizontal, ensuring that the hands were held at heart level.

Microcirculatory volume was measured with a MedaSonics model PA13 infrared photoplethysmograph (PPG; MedaSonics, CA, USA). The technique is based on changes in the intensity of transmitted light caused by the blood pulse in the tissue. Infrared light (wavelength about 850 nm) from a source in a photoelectric PPG probe is used to illuminate the skin. The light undergoes multiple scattering, absorption, reflection, and refraction in the illuminated skin and subcutaneous tissue. The emergent intensity of this light, measured by an adjacent photodetector in the emitting probe, is inversely proportional to the total blood flow in the illuminated tissue. The a/c (beat-by-beat) amplitude of the PPG configuration is directly related to the volume of the circulating erythrocytes, and is measured in arbitrary units.[22] The depth of penetration of the infrared light emitted by the PPG is characteristically less than 3 mm, and thus includes the flow in the arteriovenous shunt vessels.

Laser doppler fluxmetry is a similar technique, which uses laser light to measure the flux (velocity 3 number) of blood cells, mainly erythrocytes, in the skin by the doppler effect. A DRT-4 laser doppler fluxmeter, (LDF; Moor Instruments, Devon, UK) and 780 nm integrated probes were used in this study. Infrared light generated by a low-powered laser is directed via an optical fibre to the tissue to be studied. The back-scattered light from the tissue is collected by one or more other optical fibres, processed, and analysed. The size of the frequency shift depends on the number and velocity of the cells. Although the dominant contribution to the signal is from erythrocytes, there is also a contribution from other blood cells and from structural movements.[20,23] The critical depth of measurement of the LDF system is about 1 mm, and the measurement is therefore largely a reflection of capillary flow, although arteriovenous vessel flow may also be detected, depending on skin thickness.

We prepared the nitric-oxide-generating system by mixing two viscous solutions (A and B). In the preliminary experiment, solution A was prepared in the sterile lubricant KY jelly (Johnson & Johnson, Maidenhead, UK), to which Analar grade sodium nitrite was added to make solutions ranging from 1% to 15% (weight/volume) in a sterile plastic specimen pot. Solution B was prepared by addition of Analar grade ascorbic acid to KY jelly to make solutions ranging from 1% to 15% (weight/volume) in a separate sterile plastic pot. About 0·5 mL of each solution was separately applied to the skin of the forearms (3 cm^2) and then mixed with a sterile cotton bud. The surface reaction was stopped within a few seconds by gentle cleansing of the skin with a paper tissue.

	Microvascular volume			Microvascular flux		
	Active gel*	Placebo*	p	Active gel*	Placebo*	p
Forearm						
Baseline	98 (14)	99 (16)	>0·2	5660 (462)	4200 (325)	0·14
Gel on	1024 (130)	231 (46)	0·0002	74 800 (3940)	8990 (1370)	0·001
Gel off	944 (123)	218 (45)	0·0003	85 900 (5700)	8450 (619)	0·001
Finger						
Baseline	1100 (194)	1170 (177)	>0·2	33 400 (4200)	37 300 (5290)	>0·2
Gel on	3280 (672)	2610 (588)	0·015	108 000 (13 600)	78 300 (12 000)	0·007
Gel off	2260 (480)	2460 (648)	>0·2	86 900 (13 000)	68 000 (11 200)	0·025

Gel on=during application of gel.
Gel off=during removal of gel.
*Area under the curve (SE).

Table 2: **Comparison of active and placebo treatment in patients with severe Raynaud's syndrome**

In the main study, the microcircular responses were measured first on the forearms and then on the finger pulps in response to a 5% weight/volume mixture of solutions A and B. A similar procedure was done simultaneously on the other arm with KY jelly only, and was designated as the placebo treatment.

The position of the measurement probes was accurately marked on the skin to allow their precise reapplication when the nitric-oxide-generating and placebo gels were applied and removed. In the preliminary study, after equilibration was achieved, forearm microcircular flux was measured by LDF for 5 min after application of the gel mixture. In the main study, after an equilibration period of 15 min, assessment of the microcirculatory volume (PPG) and microcirculatory flux (LDF) was done simultaneously at adjacent sites on the centre of the volar surface of the skin of the forearm (a low-flow area with few arteriovenous shunts).[24] Measurements were made at baseline, for 10 min immediately after the simultaneous application of the gels (nitric-oxide-generating gel and placebo gel on opposite arms) and 10 min after the removal of the gels. After a recovery period of 1 h, the assessment was repeated in the main study on the pulps of the index and middle fingers (a high-flow area with many arteriovenous shunts). The arm or finger to which the active gel was applied was decided by means of a sequence of random numbers. Random numbers were generated by a third party with a forced square blocking system with alternate arms.

All data were collected and recorded by means of the Collect data acquisition program (version 1.0) via a 1401 analogue-to-digital converter interface onto computer. Waveform analysis was done off-line with a Spike-2 data analysis program and custom scripts (version 3.1). The waveforms were averaged for 60 s intervals to provide data for subsequent analysis.

Statistical analysis

The number of patients required to obtain statistical power was difficult to calculate because of the lack of previous studies. However, the uncontrolled pilot study allowed a preliminary power calculation, which indicated that a sample size of ten patients should show a difference in primary outcome measures between active and placebo treatment (α=0·05; 1-β=80%). Empirically, we chose 20 patients with severe Raynaud's syndrome and ten healthy controls.

In the main study, all patients and controls received both active and placebo treatment simultaneously for both forearm and finger assessments. All analyses and summaries were done with Microsoft Excel 5·0a and Minitab 11.2 statistical analysis packages. Areas under the curve were calculated for each individual for the following three periods: baseline, during application of active or placebo gel, and during removal of the gel. Comparisons were made between active and placebo treatments in patients and controls. Further comparisons were made between the healthy volunteers and the patients at baseline and by percentage change from baseline after application of the active gel.

The data were not normally distributed, as confirmed by probability plots for all of the data. Therefore non-parametric analysis was done with Wilcoxon's matched-pairs signed-rank sum test. A p value of less than 0·05 was taken as significant.

Results

Figure 1 shows how the study groups were derived and the randomised allocation of active and placebo gels. Demographic details of the two groups in the main study are shown in table 1. No measured or reported adverse effects were noted at the time of the investigation or when individuals were interviewed 1 week later.

Placebo treatment had no effect on microcirculatory volume (PPG) or flux (LDF) in the forearms of patients or controls (tables 2, 3). However, when the active gel was applied to the forearm, both groups showed a large vasodilator response in both microcirculatory volume and flux. The increase in blood flow reached a plateau during application of the gel, and was sustained, albeit at a lower level, after removal in both groups (figures 2,3).

In the patients, application of the active gel to the finger pulp caused a significant increase in microcirculatory volume ($p<0·05$; figure 2), which returned rapidly to the baseline volume on removal of the gel. Active gel also significantly increased finger microcirculatory flux ($p<0·01$) to a value similar to the baseline values in healthy controls. This increase was sustained, although at a lower level, after removal of the gel ($p<0·05$; figure 2). In the control group, active gel had no significant effect on finger microcirculatory volume; however, microcirculatory flux increased significantly and remained raised after removal of the gel ($p<0·01$; figure 3).

	Microvascular volume			Microvascular flux		
	Active gel*	Placebo*	p	Active gel*	Placebo*	p
Forearm						
Baseline	85 (19)	76 (10)	>0·2	4420 (435)	4040 (540)	>0·2
Gel on	1020 (60)	153 (22)	0·005	84 500 (7000)	7330 (1040)	0·005
Gel off	833 (58)	137 (22)	0·005	79 100 (9280)	7880 (1110)	0·005
Forearm						
Baseline	2380 (441)	2590 (576)	>0·2	52 000 (8950)	44 200 (7740)	>0·2
Gel on	6160 (1160)	4580 (929)	0·11	185 000 (19 500)	72 700 (14 000)	0·005
Gel off	3860 (1050)	3880 (800)	>0·2	141 000 (25 900)	69 900 (15 000)	0·007

Gel on=during application of gel.
Gel off=during removal of gel.
*Area under the curve (SE).

Table 3: **Comparison of active and placebo treatment in healthy volunteers**

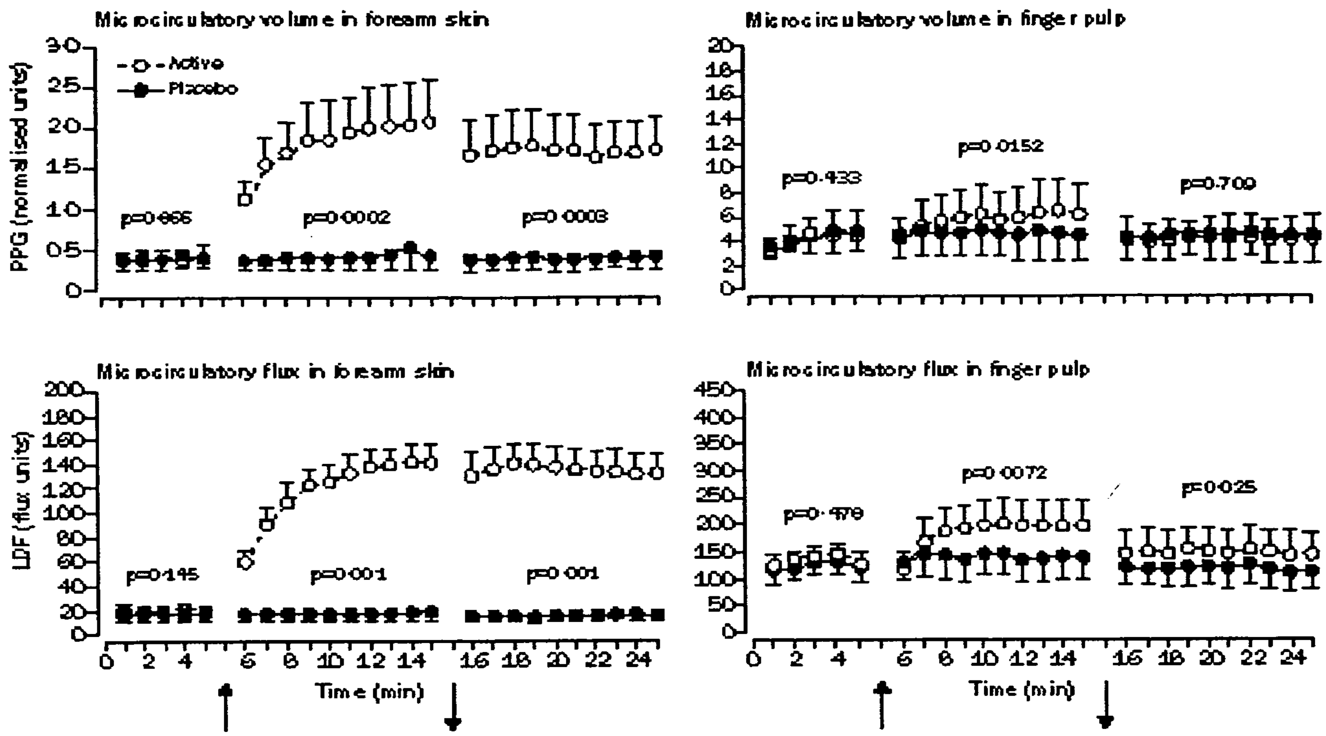

Figure 2: **Microcirculatory volume and flux in forearm skin and finger pulp in patients with severe Raynaud's syndrome**
↑=application of gel; ↓=removal of gel; points=mean; error bars=95% CI
Note scales differ for forearm skin and finger pulp.

Baseline microcirculatory volume and flux in the forearm were similar in both groups, whereas in the finger, baseline microcirculatory volume and flux were significantly lower in the patients with Raynaud's syndrome than in controls ($p<0{\cdot}01$). However, the percentage increase in microcirculatory flux in the fingers of patients was similar to that observed in the healthy volunteers.

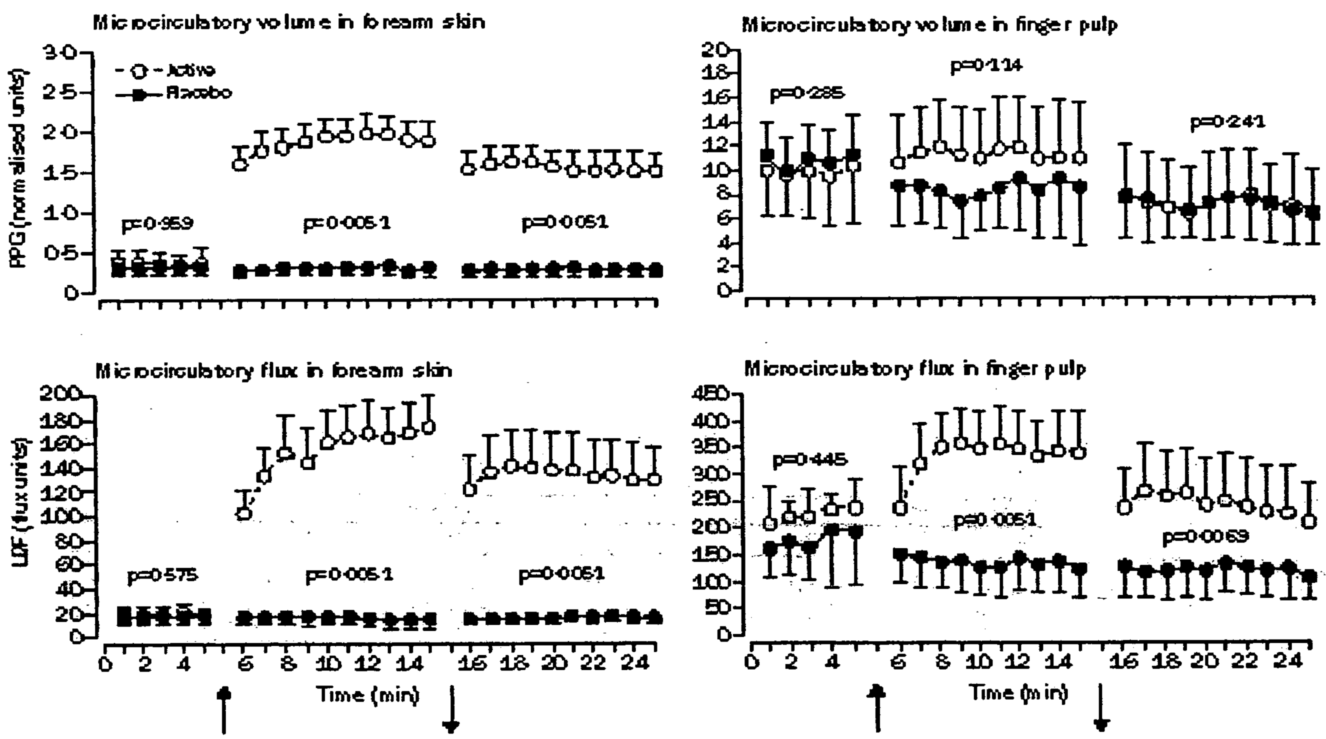

Figure 3: **Microcirculatory volume and flux in forearm skin and finger pulp in healthy volunteers**
↑=application of gel; ↓=removal of gel; points=mean; error bars=95% CI
Note scales differ for forearm skin and finger pulp.

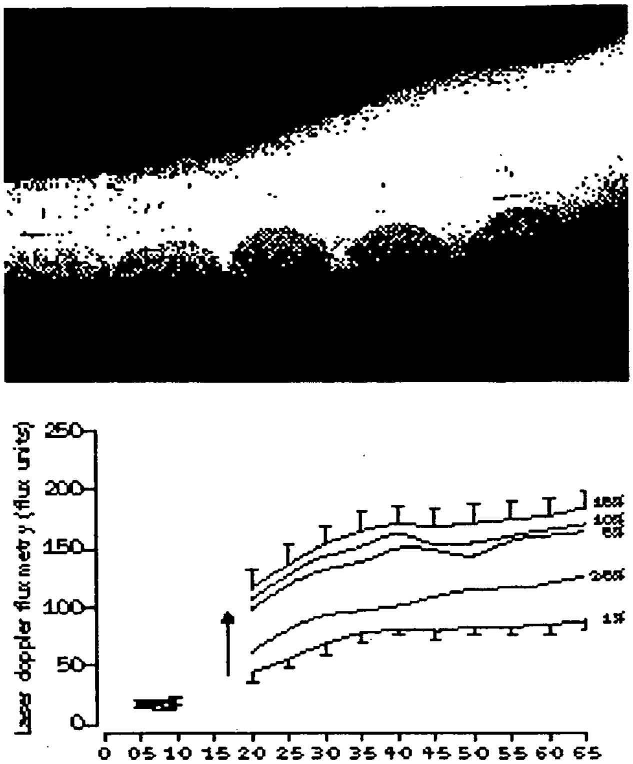

Figure 4: **Dose response of nitric-oxide-generating system**
Photograph shows forearm hyperaemic response to increasing concentrations of active gel in a healthy volunteer. Graph shows mean (95% CI) microcirculatory flux response to increasing concentrations of active gel in ten healthy volunteers. Arrow=application of active gel.

The response to the nitric-oxide-generating system was dose-dependent in terms of erythema and microvascular flux (figure 4), and correlated well with the vascular response to a warm stress test (immersion of the hand for 5 min in a water-bath maintained at 40°C) which creates maximum vasodilatation (figure 5).

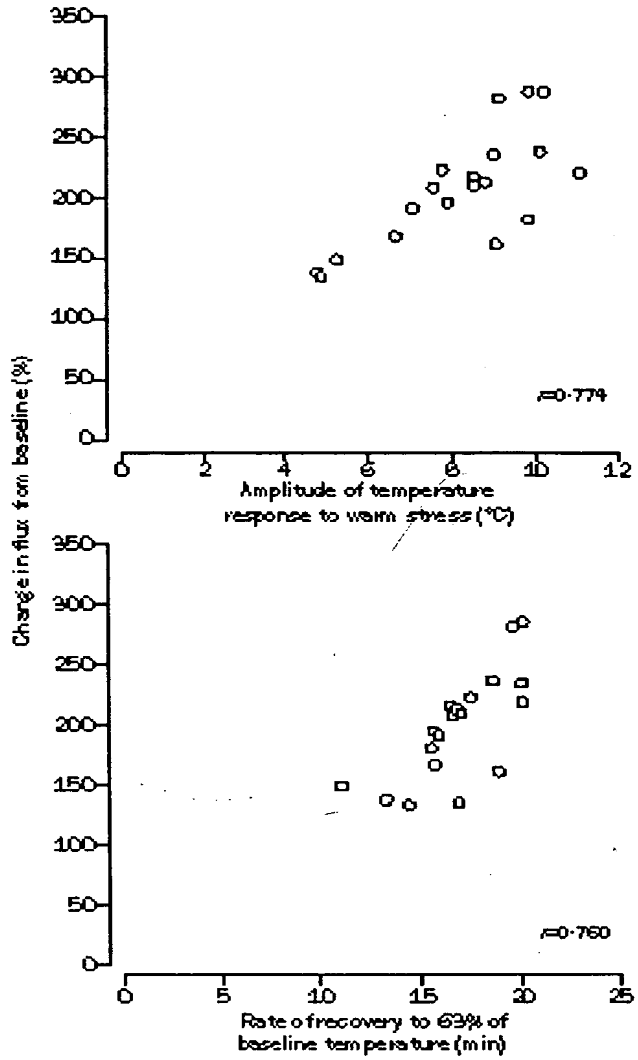

Figure 5: **Relation of warm stress test to response to active gel**

Discussion

The range of clinical presentation of digital vasospasm in patients with Raynaud's syndrome varies from mild symptoms of short duration, seen in most patients, to critical or near-critical digital ischaemia in a few. Many patients require only guidance on stopping cigarette smoking and on strategies to protect their fingers from cold exposure—eg, the use of thermal and electrically heated gloves or hand warmers. For patients with more severe disease, various vasodilatory drugs have proved only partly effective in clinical trials.[25-28] Calcium-channel antagonists, such as nifedipine, decrease the frequency, severity, and duration of attacks and the occurrence of digital lesions[29] without enhancing digital blood flow.[30] Nifedipine is generally accepted as the therapy of choice for severe cases and may be effective in cases with organic vascular wall change, such as systemic sclerosis.[6] Side-effects including headache, hot flushes, and leg oedema occur commonly even at low doses, and affect about a third of patients.

When ischaemia is severe, intravenous infusions of prostanoids and their analogues have produced sustained increases in digital blood flow and mean hand temperature, and decreased digital peripheral vascular resistance.[30,31] Slow-release, orally administered formulations are being assessed for efficacy and tolerability, although initial results have not been encouraging.[21]

Our findings accord with previous observations that Raynaud's syndrome is associated with endothelial dysfunction, in particular a decrease in nitric oxide synthesis or activity.[8] However, this may not be the only deficiency. Abnormal concentrations of endothelin-1 and calcitonin-gene-related peptide, and abnormalities of fibrinolysis, platelet aggregation, and digital-artery adrenoceptor populations have also been proposed.[32-35] Despite the range of management strategies available, most patients require a simple treatment that is reliably effective when vasospasm is most severe. The application of a nitric-oxide-generating gel at the appropriate time seems to meet this requirement.

We have previously demonstrated that the thermoregulatory response of the hands to warm stress testing can be used to assess morphological vascular changes and dysfunction of the peripheral nervous system and endothelium.[19] The correlation between the vasodilator effect of the nitric-oxide-generating system and the vascular response to the warm stress test indicates that patients with more profound functional abnormalities that show a decreased vasodilatory response to warm stress will

also show a decreased response to the topical application of the nitric-oxide-generating gel. However, our findings suggest that therapy with the gel may approach a maximum vasodilatory effect at a single application.

Nitric oxide applied to the skin in healthy individuals causes an increase in dermal blood volume and flux, as measured by standard non-invasive techniques. Nitric oxide seems to be able to diffuse 1–3 mm into the dermis to relax the vascular smooth muscle in precapillary sphincters without being oxidised by haemoglobin or superoxides. The depth of penetration is indicated by the operational characteristics of the non-invasive techniques used in the study.[20,22,23] The effects of nitric oxide on forearm blood flow and volume were large (inducing ten-fold and six-fold increases, respectively), whereas those in the finger were more modest (120% increase in volume [not significant]; 170% increase in flux, $p<0.01$). The difference in vasodilator response in these two types of skin may be due to the higher baseline flow in the finger circulation. Alternatively, the thicker epidermis of the finger pulp may present a barrier to diffusion of nitric oxide.

The forearm skin blood flow showed similar responses in patients with severe Raynaud's syndrome and in healthy volunteers, suggesting a preserved response to nitric oxide in non-affected vessels in these patients. In the finger skin of patients, although the maximum response did not equal that of the control group, the percentage increase in microcirculatory flux from baseline was similar. This finding suggests that nitric oxide can reverse the abnormal constrictor tone in diseased vessels, which may have impaired endogenous nitric oxide synthesis or activity. The smaller effect in those with more profound structural changes is consistent with this idea.

Other advantages of the gel are that it can be used at the time of attack, and that it seems to have no adverse effects. Advantages over glyceryl trinitrate cream are the absence of any systemic effect, the lack of local adverse effects, and the improbability of the development of tolerance.[36] Disadvantages are that, in its current form, the treatment is messy and tends to sting if applied to broken skin.[14] Although the length of sustained vasodilatation is unknown, it may be related to the severity of the disease; further studies into this possibility are needed. Application of our nitric-oxide-generating system may help attenuate vasospasms associated with severe Raynaud's syndrome, and may prove useful in other disorders of digital ischaemia.

Contributors

A Tucker did the studies and wrote the paper; R Pearson designed the study and helped prepare the paper; E Cooke designed the study and developed the methods used; and N Benjamin had the initial idea for the study, contributed to the design, and helped write the paper.

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